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A New Method to Determine *Giardia* Cyst Viability: Correlation of Fluorescein Diacetate and Propidium Iodide Staining with Animal Infectivity

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The viability of *Giardia muris* cysts was studied with the fluorogenic dyes fluorescein diacetate (FDA) and propidium iodide (PI). *G. muris* cysts were seen to fluoresce intensely green with FDA at an excitation wavelength of 450 to 490 nm. Cysts stained with PI fluoresced bright orange at an excitation wavelength of 450 to 490 nm and bright red at 545 to 546 nm. Examination of isolated *G. muris* cyst preparations stained with FDA-PI revealed that greater than 85% of the cysts stained green with FDA and less than 15% stained orange-red with PI. Using the mouse model for giardiasis, we inoculated FDA- or PI-stained cysts into neonatal mice. Feces were examined at days 3, 5, 8, and 11 postinoculation for the presence of cysts. Using 1,000 FDA-stained cysts as the inoculum, we detected cysts at days 5, 8, and 11 postinoculation in 19 of 19 mice, whereas a 50-fold greater dose of cysts produced infection in 27 of 27 mice at day 3 as well as at days 5, 8, and 11 postinoculation. Inoculation of mice with either 5,000 or 50,000 PI-stained *G. muris* cysts did not produce infection in any of the animals. Necropsy of mice infected with FDA-stained cysts showed trophozoites within the intestines. No trophozoites were detected within animals inoculated with PI-stained cysts. These results demonstrate that FDA-positive cysts are viable, as determined by infectivity, while PI-positive cysts are nonviable and incapable of producing *G. muris* infections in vivo. The development of this rapid and inexpensive test should facilitate testing of the effects of various chemical agents and environmental factors on the viability of *Giardia* cysts.

Methods used for determining the viability of *Giardia* cysts have included dye exclusion (2, 12), excystation in vitro (2, 3, 12, 18), and animal models for infectivity (19). In dye exclusion methods, those cells that do not respire, glycolyse, or extend cellular processes when maintained in a tissue culture system have been shown to be stained with this technique (15). Viable, living cells remain unstained. However, vital dyes have been shown to have a greater affinity for proteins in solution than for nonliving cells (17) and may be toxic at low concentrations (22). In addition, vital dye results correlated poorly with other criteria when used to demonstrate *Giardia* cyst viability (2, 12). Excystation would seem to be an indicator of the viability of *Giardia* cysts, since trophozoites must be released from the cysts to infect the host, but the broad definition of excystation used (2, 18), which even includes aborted attempts at trophozoite emergence from the cysts, makes this method subjective and likely to overestimate true cyst viability. Models of animal infectivity require the use of cyst inocula with numbers extending up to several thousand. This technique may provide information about the viability of groups of cysts but reveals little pertaining to individual cysts. In addition, all of these methods are indirect tests in that the individual cysts tested are lost for further experimentation.

Since described in 1966 by Rotman and Papermaster (20), fluorogenic dyes have been accepted as a sensitive means for determining cell viability in a variety of cell types ranging from protozoa to mammalian cells (10). This study was designed to determine the efficacy of the fluorescein diacetate (FDA)-propidium iodide (PI) method for measuring the viability of *Giardia muris* cysts. Animal infectivity was used as an in vivo indicator of the viability of cysts stained with fluorogenic dyes. Using the mouse model for giardiasis developed in 1976 by Roberts-Thomson et al. (19), we gave neonatal mice cyst inocula consisting of either PI-positive cysts or FDA-positive cysts. Fecal cyst shedding was used as an indicator of established infection and correlated with *G. muris* viability, as indicated by the incorporation of fluorogenic dyes.

MATERIALS AND METHODS

The staining solutions were made by the method of Jones and Sneft (11). Briefly, a stock solution of FDA was made by mixing 10 mg of FDA with 1 ml of acetone. A working solution was prepared by adding 0.04 ml of stock solution to 10 ml of Dulbecco phosphate-buffered saline at pH 7. A stock solution of PI (Sigma Chemical Co., St. Louis, Mo.) was made by mixing 0.5 mg of PI with 50 ml of Dulbecco phosphate-buffered saline at pH 7. Pellets of *G. muris* cysts were stained with these dyes at concentrations of either 4 μg of FDA per 10^6 cysts or 3 μg of PI per 10^6 cysts. Cysts were stained for 5 min before microscopic observation. Stained slides were examined with an Olympus BH-2 epifluorescence microscope at excitation wavelengths of 455 to 490 nm for FDA and 545 to 546 nm for PI.

G. muris cysts were isolated from the feces of infected mice by the procedure of Roberts-Thomson et al. (19). CF-1 non-Swiss mice (Harlan Sprague-Dawley) (14 to 21 days old) were inoculated intragastrically with 0.1 ml of a PI- or FDA-positive *G. muris* cyst suspension. Nine mice per litter

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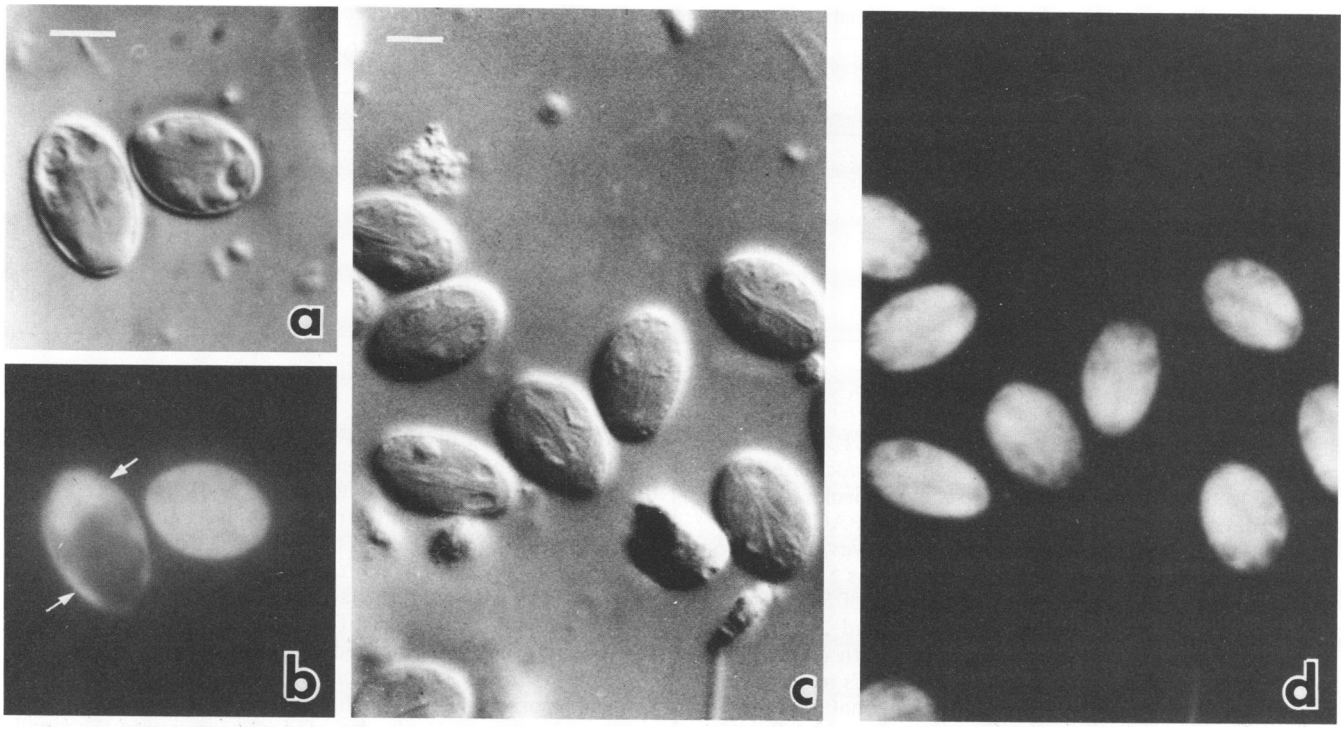

FIG. 1. (a) Differential interference contrast (DIC) micrograph of *G. muris* cysts determined by FDA incorporation (see panel b) to be viable. Bar, 5 μm (for panels a and b). (b) Fluorescence micrograph of the same *G. muris* cysts shown by DIC in panel a. Cysts were labeled with the fluorogenic dye FDA and fluoresced intensely green at an excitation wavelength of 450 to 490 nm. Intense fluorescence was detected within the trophozoite cytoplasm and also in the space between the trophozoite and the cyst wall (arrows). (c) DIC micrograph of *G. muris* cysts determined by PI incorporation (see panel d) to be nonviable. Bar, 5 μm (for panels c and d). (d) Fluorescence micrograph of the same *G. muris* cysts shown by DIC in panel c. Cysts were heated at 80°C for 15 min and then labeled with PI; they fluoresced bright orange at an excitation wavelength of 450 to 490 nm.

were given the appropriate inoculum. One animal per litter served as a control and was not inoculated.

Prior to inoculation, fecal exams had been performed daily on each animal for a period of 4 days to ensure that the animals did not have an endogenous infection. Also, one animal per litter was sacrificed to perform a microscopic examination of the mucosa for *G. muris* trophozoites.

Fecal examinations of individual animals were carried out on days 3, 5, 8, and 11 postinoculation with both zinc sulfate flotations and direct-smear fecal examinations. At the end of the experimental trial period, an animal from each group was sacrificed to microscopically check for the presence of trophozoites in the small intestine.

Cysts for the FDA incoculum were freshly isolated and stained with FDA-PI. The cyst inoculum number in one series of experiments was determined by the average number of FDA-positive cysts from four separate aliquots of the cyst suspension. In two separate experiments with FDA-positive cysts obtained in this manner and counted with a hemacytometer, 1,000 cysts per animal and 50,000 cysts per animal were administered. In the other experimental series, aliquots of FDA-positive cysts were obtained with a fluorescence-activated cell sorter (FACS IV; Becton Dickinson and Co., Paramus, N.J.), and 1,000 cysts per animal were administered. All cysts were inoculated intragastrically via polyethylene tubing with a 1-ml syringe.

Cysts for the PI inoculum were obtained with an FACS IV and also through experimental induction by immersing cysts (500,000/ml) in a warm water bath at 80°C for 15 min. Microscopic examination of aliquots of both cyst suspensions showed all cysts to be PI positive. In one experimental group, 5,000 PI-positive cysts, obtained from the FACS IV, were administered to each animal. Another experimental group of mice was inoculated with 50,000 PI-positive cysts induced by heating.

RESULTS

Isolated cysts of *G. muris* were identified morphologically by light microscopy based on the following characteristics: shape, size, the presence of two to four nuclei usually in a polar position, and the appearance of linear structures representing flagellar axonemes (Fig. 1a and c). *G. muris* cysts incubated in the fluorogenic substrate FDA were seen to fluoresce intensely green (Fig. 1b). PI-stained cysts, when viewed with the excitor-barrier filter combination used for FDA (450 to 490 nm), were easily distinguishable from FDA-stained cysts as they fluoresced bright orange (Fig. 1d). Examination of PI–stained cysts at 545 to 546 nm revealed an intense red fluorescence. Investigation of routine *G. muris* cyst isolates (greater than 50) indicated that 85 to 90% of the cysts stained green with FDA, while less than 15% stained orange-red with PI. Occasionally, we observed *G. muris* cysts that did not metabolize FDA or intercalate PI and thus appeared black. Heating *G. muris* cysts for 15 min at 80°C converted all cysts to PI positivity (Fig. 1c and d).

The viability of *G. muris* cysts was investigated by the administration of dye-exposed cysts to neonatal mice (Table 1). Control neonatal mice that did not receive an inoculum of *G. muris* cysts did not pass cysts in their feces during the

TABLE 1. *G. muris* cyst viability: correlation between fluorogenic dye incorporation and infectivity in neonatal mice

No. of cysts inoculated per animal (no. of animals)	Fluorescent substrate and appearance[a]	Method of cyst isolation	*G. muris* cyst appearance on indicated day postinoculation:			
			3	5	8	11
Control: 0 (n = 8)	None	None	0	0	0	0
Experimental						
1,000 (n = 9)	FDA positive	Sucrose density gradient	0	9	9	9
50,000 (n = 27)	FDA positive	Sucrose density gradient	27	27	27	27
50,000 (n = 40)	PI positive (induced)	Sucrose density gradient	0	0	0	0
1,000 (n = 10)	FDA positive	FACS IV	0	10	10	10
5,000 (n = 10)	PI positive	FACS IV	0	0	0	0

[a] As determined by fluorescence.

course of the experiments (3, 5,8, and 11 days postinoculum). None of the mice experimentally inoculated with doses of 1,000 FDA-positive *G. muris* cysts, isolated with a sucrose density gradient (n = 9) or with the FACS IV (n = 10), shed cysts on day 3 postinoculation. However, 100% of the mice were positive for fecal cysts on days 5, 8, and 11. All of the mice infected (n = 27) with doses of 50,000-FDA positive cysts passed cysts on days 3, 5, 8, and 11 postinoculation. Necropsy of the animals infected with either 1,000 or 50,000 FDA-positive cysts revealed *G. muris* trophozoites within the small intestines. Inoculation of neonatal mice with FDA-positive cysts resulted in *G. muris* multiplication within the intestines and subsequently the shedding of cysts in feces, with a slight difference in the day of onset of infection. Mice receiving 1,000 FDA-positive cysts did not shed cysts on day 3, while those receiving 50,000 FDA-positive cysts were positive on day 3, presumably reflecting the 50-fold difference in cyst numbers in the inocula. A total of 10 mice were each inoculated with 50,000 PI-positive cysts isolated with the FACS IV, while in another experimental series in which heat was used to induce PI staining, 40 mice were each inoculated with 50,000 PI-positive cysts. None of the mice inoculated with PI-positive *G. muris* cysts shed in their feces at any time during the experiments. Examination of the intestinal contents from each litter at necroscopy (n = 5) did not reveal any *G. muris* trophozoites in representative animals. Inoculation with PI-positive cysts failed to establish an infection, as evidenced by the lack of *G. muris* cysts in the feces and the absence of trophozoites within the small intestines.

DISCUSSION

Our studies have shown that the fluorogenic dyes FDA and PI can be used as an in vitro test to determine the viability of *G. muris* cysts. The viability of cysts stained with FDA was established by demonstrating that these cysts were capable of infectivity in 100% of the animals tested (n = 46) in a neonatal mouse system, whereas nonviable cysts stained with PI never produced an infection (n = 50). Using the FACS IV to sort cysts specifically stained with either FDA or PI or using cysts isolated with a sucrose density gradient, we demonstrated that only cysts stained with FDA were capable of producing an infection, as evidenced by fecal cyst shedding. In addition, PI-positive cysts, whether sorted in the FACS IV (cysts naturally stained with PI) or experimentally induced (by heat) to stain with PI, never produced an infection, even at doses that were 50-fold higher than the dose that produced an infection with FDA-positive cysts and 500 times the dose reported to produce an infection in mice (19). Large doses of either FDA- or PI-stained cysts were administered to animals in this study. However, the use of dose-response experiments with FACS IV-isolated FDA-positive cysts may permit a more precise determination of the minimum infective dose of *G. muris*. Nonetheless, the striking correlation between the uptake of FDA and animal infectivity together with the lack of it for PI-stained *G. muris* cysts clearly indicated that FDA cysts were viable, while PI cysts were not, as determined by animal infectivity.

Further support for the correlation of cyst viability with the incorporation of FDA lies in the fact that *G. muris* trophozoites were always detected at necropsy in the small intestines of mice infected with FDA-positive cysts but were never encountered in the small intestines of mice inoculated with PI-positive cysts. In addition to animal infectivity, the excystation of *Giardia* cysts has also been established as a criterion for cyst viability (2, 3, 16, 18). We have observed (unpublished observation) FDA-positive cysts that underwent excystation and yielded free-swimming, motile trophozoites which were FDA positive. Similar results have never been observed for PI-stained cysts. A subpopulation of *G. muris* cysts that did not directly stain with FDA or PI was observed and appeared black. These nonstaining cysts were considered to be a subpopulation of the FDA-staining cysts since (i) black cysts were seen to convert to green fluorescence during microscopic examination, (ii) upon storage the proportion of black cysts decreased while the proportion of FDA-stained cysts increased and the proportion of PI-stained cysts remained the same, and (iii) inoculation of mice (n = 10) with nonstaining cysts isolated with the FACS IV resulted in infection (unpublished observations). Collectively, these data, determined by animal infectivity and excystation, both of which are established criteria for *Giardia* cyst viability (2, 3, 12, 18, 19), have demonstrated the efficacy of the fluorogenic dyes FDA and PI in determining the viability of *G. muris* cysts.

Fluorogenic dyes have been accepted as a sensitive means for the determination of cell viability (20). Fluorogenic dyes are molecules that are nonfluorescent and can serve as substrates in certain enzymatic reactions. Nonspecific intracellular esterases cleave the two acetate groups via their ester linkage in the nonpolar substrate FDA and have been shown to yield the polar molecular fluorescein, which fluoresces at an excitation wavelength of 450 to 490 nm (8). It has been established that an intact lipid bilayer slows the leakage of the fluorochrome from within intact cells, while injured cells cannot retain or accumulate the fluorochrome (20). In *G. muris* cysts fluorescein was observed to accumulate both within the trophozoite and within the space between trophozoite membrane and cyst wall (Fig. 1b). This observation

suggests that in viable *Giardia* cysts both the bilipid membrane of the cell and the cyst wall may serve as barriers to the diffusion of fluorescein, whereas damaged or nonviable cysts may not accumulate fluorescein owing to the presence of disrupted membranes. Reports (7, 20) have implicated lipase, acylase, and proteinase in the hydrolysis of FDA, but the presence of these enzymes has not been investigated in *Giardia* trophozoites or cysts. Experimentation has shown FDA to be nontoxic to a wide variety of cell types, ranging from primitive eucaryotes to mammalian cells and tissues in culture (20). The fluorogenic dye PI has not been known to traverse intact cell membranes; therefore, only cells with disrupted or broken membranes are counterstained by PI (9, 13). The phenanthridinic fluorochrome PI has been shown to be specific for double-stranded nucleic acids and, upon intercalation, resulted in approximately a 25-fold increase in fluorescence emission at about 610 nm, with the maximum excitation peak at 530 nm (1). PI has been shown to be nontoxic at up to 10 times the concentration used in this study (11).

The use of fluorogenic dyes to investigate the viability of *Giardia* cysts should have several advantages over previously used methods, including dye exclusion (12), excystation (2, 3, 12, 18), and animal infectivity (19). First, the fluorogenic staining procedure is simple and inexpensive and requires only a few minutes to complete. However, the method must be carefully performed to obtain optimum staining. Particular attention should be paid to the concentration of dye used as a function of cell number and also the proper storage of stock or working solutions. A second advantage of using fluorogenic dyes is that direct results on the viability of cysts can be obtained on groups of cysts and individual cysts, which can then be subjected to further experimentation without compromising the sample. Third, the use of fluorogenic dyes should lend itself readily to the analysis of subpopulations of *Giardia* cysts by flow cytometry, since both FDA and PI have been successfully used for this purpose in a variety of cell types (4, 21). Last, the effects of disinfectants and various environmental factors on the viability of *Giardia* cysts can be tested with fluorogenic dyes on the same population of cysts both before and after exposure to chemical or physical agents.

In conclusion, we have developed a method based on the use of fluorogenic dyes that is potentially useful for investigating the viability of *Giardia* cysts in vitro. This method should enable us to recover and thus characterize viable and nonviable *Giardia* cysts. Although it is assumed that *Giardia* cysts from a variety of species are similar, some differences have been noted in excystation (16); therefore, the application of fluorogenic dyes to cysts of different host origins or morphological types (5, 6, 14) may provide interesting information on cyst viability, morphology, and metabolism.

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